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AN IMPROVED METHOD FOR THE DETERMINATION OF PANTOTHENIC ACID IN TISSUES

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During work on coenzyme A (CoA), a method for the liberation of pantothenic acid from the coenzyme was worked out (1). The method involves the use of a combination of two enzymes; namely, intestinal phosphatase and an enzyme extractable from acetone powder of bird liver (chicken (2), duck, or pigeon). This procedure proved the most efficient pretreatment to liberate bound vitamin under conditions in which taka-diastase, papain, or mylase P, enzymes which are usually employed to release bound pantothenate, are unsuccessful. Neilands and Strong (2) have applied this method to the measurement of pantothenate in various biological products and were able to demonstrate a 4- to 10-fold greater yield of pantothenate than by the use of the earlier methods for estimating bound pantothenate.

A considerable inconvenience in the use of the combined enzyme method, already apparent in the earlier work (1), was the relatively high pantothenate blank introduced by the enzyme preparations, in particular the liver enzyme. This high blank necessitates applying a correction factor, which in the case of materials of low pantothenate content may represent up to about 100 per cent of the pantothenate present in the test material. This high enzyme blank is due to the relatively high CoA content in the liver enzyme, which, during the incubation period, is converted to pantothenate.

Recently a method for reducing the CoA content of the liver enzyme has been developed, which is based on the observation, in this laboratory (3), that CoA can be very efficiently adsorbed by various resins as well as by charcoal. It appeared that by treatment with one of these resins the CoA content, and therewith the blank of the liver enzyme, can be reduced to a very low level without appreciable loss of CoA-splitting activity. The present report is a description of the application of resin treatment to the preparation of liver enzyme and its use in liberating bound pantothenate.

Enzyme Preparations

Liver Enzyme—This enzyme was prepared from acetone-dried pigeon liver powder as previously described (1). The enzyme can also be pre-

pared from chicken or duck liver acetone powders. Through Armour and Company, pigeon liver acetone powder is now made commercially available. A sample of this material was tested and found to yield a potent preparation of the enzyme.

The enzyme solution is prepared by carefully rubbing the acetone powder into 10 times its weight of ice-cold 0.02 M potassium bicarbonate, to give a smooth suspension. After centrifuging at 3000 r.p.m. for 30 minutes in the cold, the reddish brown supernatant is saved and stored at -20° .

Treatment of Liver Enzyme with Dowex-1—Dowex-1 (anion exchange resin), 200 to 400 mesh, obtained from The Dow Chemical Company, Midland, Michigan, is washed twice with 10 volumes of N HCl by centrifugation. The acid-treated resin is then washed eight to ten times with 10 volumes of water until the pH is approximately 5. The resin is left as a slurry of a consistency which can just be pipetted with a 10 ml. serological pipette.

To 1 volume of acid-washed Dowex-1 are added a few drops of M tris(hydroxymethyl)aminomethane buffer, pH 8.3, to bring the pH to about 8.0. Then 1 volume of ice-cold liver enzyme is added and the suspension is stirred for 3 to 5 minutes in an ice bath. The suspension is centrifuged at 5000 r.p.m. and the supernatant is poured off and again treated with 1 volume of Dowex-1 adjusted to pH 8.0. Two such Dowex treatments usually reduce the CoA to a very low level as judged by pantothenate determination (cf. Table I).

Intestinal Phosphatase—Enzyme solutions were prepared from a product available from Armour and Company. A 2 per cent solution of their product is prepared in distilled water and tested for phosphatase activity by the method of Schmidt and Thannhauser (4). This solution generally contains around 100 Schmidt-Thannhauser units per ml., and 0.1 ml., containing 5 to 10 units, is used in the test system.

CoA was estimated by the method of Kaplan and Lipmann (5).

Pantothenic acid was measured by the turbidimetric method of Skeggs and Wright (6), with *Lactobacillus arabinosus* 17-5 as test organism.

Pantothenic Acid Assay

Liberation of Bound Pantothenate with Dowex-Treated Enzymes—The routine method for liberation of pantothenate bound as CoA is as follows:

To a sample of the unknown containing 5 to 15 γ of bound pantothenate are added 0.1 ml. of intestinal phosphatase (containing 5 to 10 units (4)), 0.2 ml. of Dowex-treated liver enzyme, and 0.1 ml. of M tris(hydroxymethyl)aminomethane buffer, pH 8.3, in a final volume of 1.0 ml. A control tube without the sample, but containing the two enzymes, is also prepared. The tubes are incubated for 3 hours at 37° , when the reaction is stopped by boiling. The samples are then diluted to bring them within

range of the microbiological assay. With *L. arabinosus* as test organism and a turbidimetric assay, pantothenate is best detected in amounts of 0.02 to 0.08 γ . If the unknown contains 5 to 15 γ of bound pantothenate, then a dilution of 1:100 to 1:300 will permit the microbiological assay. This dilution will be large enough to minimize the blank value of the enzymes and therefore an unduly large correction factor is avoided.

Comments

Removal of CoA by Dowex-1—Conventionally prepared liver enzyme, alone and with CoA, was incubated before and after treatment with the resin. The data of Table I show that two resin treatments reduce the blank 9-fold without impairing liberation of pantothenic acid.

As an example for a determination on pantothenic acid-poor material,

TABLE I
Effect of Dowex Treatment on Pantothenate Content of Liver Enzyme

	Pantothenic acid per 0.2 ml. enzyme*	Pantothenic acid liberated from 20 units CoA†	
	γ	γ	per cent
Fresh Armour enzyme, Lot 5.....	4.4	13.2	95
1st Dowex treatment.....	1.3	13.1	94
2nd " ".....	0.5	13.9	99

* For this test, the enzymes were incubated in the absence of CoA.

† Each tube contained 0.1 ml. of intestinal phosphatase, 20 units of CoA, 0.1 ml. of tris(hydroxymethyl)aminomethane buffer, pH 8.3, and 0.2 ml. of pigeon liver enzyme in a final volume of 1.0 ml.

an assay on an extract from rice hulls is described. 300 mg. of rice hulls were suspended in 9.0 ml. of water, boiled 15 minutes, centrifuged, and the supernatant was concentrated to 2.8 ml. 2.0 ml. of this concentrated extract were treated with the combined enzymes by the method used in Table I. After the 3 hour incubation period, the samples were diluted 1:10 for the microbiological assay. The corrected value for pantothenic acid was 2.35 γ per ml. of concentrated hull extract. Even with the relatively low pantothenic acid content, the blank was only 6 per cent of the total value. A comparison with the high blank for untreated liver extract (Table I) shows that with such extracts the blank would have amounted to such a large percentage of the total that it would have seriously vitiated the determination.

Function of Liver Enzyme in Pantothenic Acid Liberation

As previously noted, alkaline phosphatase removes all bound phosphate from the coenzyme. The dephosphorylated split-product is, however, with

L. arabinosus still only one-tenth to one-fifth as active as free pantothenate. To complete the liberation of pantothenic acid, the action of the liver enzyme is required (7). The link in the coenzyme, acted upon by the liver enzyme, was suspected to be a peptidic link (8). Recent studies have clarified this point. The discovery of the *Lactobacillus bulgaricus* factor (LBF) (9) and its identification as a CoA fragment (10) have cast new light on the nature of the functioning of the liver enzyme in pantothenic acid liberation: Snell and his group (10) observed that intact coenzyme A was inactive as LBF, but that this factor was liberated from CoA by intestinal phosphatase. On the other hand, treatment with liver enzyme inactivated the CoA for activity as LBF. In parallel studies in this laboratory, it appeared that intestinal phosphatase left the pantothenic acid still linked to a sulfur-containing moiety (8), which eventually

TABLE II
Liberation of Pantothenate from L. bulgaricus Factor (LBF)

	Treatment	Pantothenic acid per mg.	Bound pantothenic acid
LBF*	None	γ	γ
"	Dowex-liver	1.35	.
"	" + intestinal phosphatase	5.25	3.90
		5.10	3.75

* LBF concentrate from Merck and Company, Inc.; contained 100 LBF units per mg.

was removed by the liver enzyme. The final explanation of the activity of the liver enzyme was obtained by the identification by Snell *et al.* (11) of LBF as *N*-pantothenylthiolethylamine. The liver enzyme, therefore, splits the peptidic link between the carboxyl of pantothenic acid and the amino group of thiolethylamine.

An example of the liberation of pantothenate from a crude concentrate of LBF is given in Table II. It can be seen that no more pantothenate is liberated by the combined enzymes than is released by the liver enzyme alone, in confirmation of Snell's (10) observation that LBF may be derived from CoA by treatment with intestinal phosphatase. Snell *et al.* (11) report a value of 0.03 γ of pantothenate per LBF unit. By use of Snell's factor, 3.9 γ per mg. of bound pantothenate, in the sample of Table II, amounts to 130 LBF units per mg. This is in good agreement with an estimated value of 100 LBF units per mg. obtained by *L. bulgaricus* assay.¹

¹ We are indebted to Dr. David Hendlin of Merck and Company, Inc., for this assay and the LBF preparation.

DISCUSSION

During work with CoA over the past few years, it has become apparent that a variety of degradation products of the coenzyme may arise through enzymatic cleavage of CoA by certain enzymes in tissues (1). The pantothenic acid conjugate of Cheldelin and coworkers (12) and the LBF of Snell *et al.* (10) and Williams *et al.* (9) may be considered as examples of such degradation products. Other breakdown products of the coenzyme have been prepared in this laboratory by use of various enzymes (13). These split-products no longer possess CoA activity. Most of them still contain pantothenic acid bound in such a form as to be unavailable to *L. arabinosus*. However, from all the degradation products so far known, pantothenate can be liberated by the combined enzyme method. This experience in turn appears to confirm our previous assumption that most, if not all, cellular pantothenic acid is bound in CoA.

SUMMARY

1. A method is described for treatment of liver enzyme with Dowex-1 which removes bound forms of pantothenic acid. With this treatment, the ability to liberate bound pantothenic acid from various materials is not impaired.
2. The use of Dowex-treated liver extracts in the combined enzyme treatment for liberation of pantothenic acid reduces the blank in the pantothenic acid assay to a very small correction value. This makes the assay applicable to material with low pantothenic acid content.
3. The function of the liver enzyme in the assay procedure is discussed.

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